HHS Public Access

Author manuscript

Cell Signal. Author manuscript; available in PMC 2017 June 01.

Published in final edited form as:

Cell Signal. 2016 June ; 28(6): 552–560. doi:10.1016/j.cellsig.2016.02.010.

The adenosine metabolite inosine is a functional agonist of the adenosine A_{2A} receptor with a unique signaling bias

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Abstract

Inosine is an endogenous purine nucleoside that is produced by catabolism of adenosine. Adenosine has a short half-life (approximately 10 s) and is rapidly deaminated to inosine, a stable metabolite with a half-life of approximately 15 h. Resembling adenosine, inosine acting through adenosine receptors (ARs) exerts a wide range of anti-inflammatory and immunomodulatory effects in vivo. The immunomodulatory effects of inosine in vivo, at least in part, are mediated via the adenosine A_{2A} receptor ($A_{2A}R$), an observation that cannot be explained fully by in vitro pharmacological characterization of inosine at the $A_{2A}R$. It is unclear whether the in vivo effects of inosine are due to inosine or a metabolite of inosine engaging the $A_{2A}R$. Here, utilizing a combination of label-free, cell-based, and membrane-based functional assays in conjunction with an equilibrium agonist-binding assay we provide evidence for inosine engagement at the $A_{2A}R$ and subsequent activation of downstream signaling events. Inosine-mediated $A_{2A}R$ activation leads to cAMP production with an EC_{50} of 300.7 μM and to extracellular signal-regulated kinase-1 and -2 (ERK1/2) phosphorylation with an EC_{50} of 89.38 μM. Our data demonstrate that inosine produces ERKl/2-biased signaling whereas adenosine produces cAMP-biased signaling at the $A_{2A}R$, highlighting pharmacological differences between these two agonists. Given the in vivo stability of inosine, our data suggest an additional, previously unrecognized, mechanism that utilizes inosine to functionally amplify and prolong $A_{2A}R$ activation in vivo.

Keywords

Inosine; $A_{2A}R$; adenosine; signaling bias; cAMP; ERK1/2

1. Introduction

Inosine is an endogenous purine nucleoside formed by deamination of adenosine. As with adenosine, it is produced and released into the extracellular space during normal cell metabolism. Adenosine has a short half-life of less than 10 s in vivo [1] and is rapidly deaminated to inosine by adenosine deaminase. Inosine has a much longer in vivo half-life

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Conflict of Interest: The authors declare that they have no conflict of interest.

than adenosine of approximately 15 h [2]. In interstitial fluids, the basal level of inosine is in the micromolar range and can be two to seven times that of adenosine [3-6], During ischemia and sepsis interstitial inosine levels rise dramatically and in certain disease states can be greater than 1 mM [7] and remain elevated longer than those of adenosine [8], Consequently, inosine levels become markedly greater than those of adenosine under disease conditions (>10-fold; [3-6]). The biological significance of the high levels of inosine in vivo is poorly understood.

As has been observed for adenosine, inosine exerts a wide variety of anti-inflammatory and immunomodulatory effects in vivo and in vitro. These include inhibition of proinflammatory cytokine and chemokine production [9-11], stimulation of anti-inflammatory cytokine production [9], protection from TNBS-induced colitis [12], LPS-induced endotoxemia [13,14], LPS-induced acute lung injury [10], glycodeoxycholic acid-induced acute pancreatitis [15], streptozotocin-induced and non-obese type 1 diabetes [16] as well as improvement of islet transplant survival [17]. However, the molecular mechanisms underlying the anti-inflammatory and immunomodulatory effects of inosine are incompletely understood. They do not require cellular uptake of inosine, suggesting involvement of cell surface receptors in this process. However, the presence of cell-surface receptors specific for inosine has not been documented. It is widely stated that the biological effects of inosine are mediated through ligation of specific membrane-bound G protein-coupled receptors (GPCRs) termed P_1-purinoceptors, also known as adenosine receptors (AR; [18]).

There are four pharmacologically distinct adenosine receptor subtypes, termed A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R [19], $A_{2A}R$ and $A_{2B}R$ are coupled to the stimulatory G protein Gαs while A_1R and A_3R are coupled to inhibitory G protein Gi [19]. Thus, adenosine occupation at the $A_{2A}R$ and $A_{2B}R$ leads to an increase in intracellular cAMP levels, whereas adenosine ligation at the A_1R and A_3R results in a reduction in intracellular cAMP levels. Among the four AR subtypes, $A_{2A}R$ is the most effective in downregulating inflammation through modulation of intracellular cAMP levels. Although inosine is a functional agonist for A_1R and A_3R, with EC_{50} values of 290 μM and 0.25 μM, respectively, inosine-mediated anti-inflammatory effects resemble those of the activated $A_{2A}R$. In vivo studies utilizing receptor knockout mice demonstrate that the anti-inflammatory properties of inosine are mediated in part through the $A_{2A}R$ [20,21], Additionally, inosine-mediated inhibition of the production of the pro-inflammatory cytokine TNF-α following LPS-stimulation of peritoneal macrophages is partially reversed by the $A_{2A}R$-selective antagonist DMPX in vitro [9]. Nevertheless, efforts directed toward a comprehensive demonstration of inosine as a functional agonist at the $A_{2A}R$ at the molecular and cellular level or inosine's ability to bind directly to the $A_{2A}R$ have been unsuccessful. Hence, inosine is largely considered to be inactive at the $A_{2A}R$ [22] in spite of a large body of in vivo data suggesting otherwise.

To understand the molecular mechanisms underlying the inosine-mediated anti-inflammatory and immunomodulatory effects, we examined inosine agonism at both the human and mouse $A_{2A}Rs$ utilizing a variety of cell-based and membrane-based assays. Here we demonstrate that inosine not only binds to the $A_{2A}R$ but also activates $A_{2A}R$ -mediated cAMP production and ERK1/2 phosphorylation. To the best of our knowledge, this is the

first comprehensive demonstration of inosine functional agonism at the $A_{2A}R$. Our data suggest the existence of a sustained $A_{2A}R$ receptor signaling mechanism that is driven by inosine long after its more potent predecessor, adenosine, has been degraded, adding a new dimension to the anti-inflammatory and immunomodulatory role of the $A_{2A}R$.

2. Materials and Methods

2.1. Materials

NECA, CGS 21680, and ZM241385 were purchased from Tocris Biosciences. [^{3}H]CGS 21680 was purchased from PerkinElmer Corporation. Rolipram, adenosine, inosine, inosine monophosphate, xanthine, hypoxanthine, adenosine 5′ [α,β-methylene] diphosphate and dipyridamole were purchased from Sigma-Aldrich.

2.2. Cell culture

CHO-K1 cells stably expressing human $A_{2A}R$ (CHO-h$A_{2A}R$; [23]) and human $A_{2B}R$ (CHO-h$A_{2B}R$; [23]) were grown in DMEM/F-12 (1:1) supplemented with 10 % FBS, 2 mM glutamine and G418 (0.2 mg/ml). CHO-K1 cells transiently expressing mouse $A_{2A}R$ (m$A_{2A}R$) were grown in the same medium without G418. All cells were maintained at 37 °C in a 5 % CO_2 incubator.

2.3. CHO-K1 transfection

CHO-K1 cells were transiently transfected with either a pCMV6 vector bearing the m$A_{2A}R$ gene or the pCMV6 vector alone using Lipofectamine 3000 (Life Technologies) according to the manufacturer's suggested protocol. Transfected cells were cryopreserved in DMEM/F-12 (1:1) containing 5 % DMSO at −195 °C (liquid nitrogen).

2.4. Dynamic mass redistribution (DMR) assay

DMR responses were assessed using an EnSpire multimode plate reader (PerkinElmer) according to a manufacturer-suggested protocol. Briefly, CHO-K1 and CHO-h$A_{2A}R$ cells were seeded onto EnSpire-LFC, 96-well microplates (PerkinElmer) and cultured for 18 h at 37 °C in a 5% CO_2 incubator. Before the assay, cells were washed with assay buffer (HBSS with 20 mM HEPES containing either 1 % DMSO alone or 100 nM ZM 241385 and 1 % DMSO) and allowed to equilibrate for 2 h at room temperature near the EnSpire multimode plate reader. Initial baseline DMR measurements were collected for 5 min, agonists and inverse agonist were added and inducible DMR measurements were collected for an additional 15 min.

2.5. Cell-based cAMP assay

CHO-h$A_{2A}R$ [23], CHO-h$A_{2B}R$ [23] and CHO-K1 cells transiently expressing m$A_{2A}R$ were seeded in 96-well half-area white plates (Greiner bio-one; 5×10^3 cells/well for h$A_{2A}R$ and h$A_{2B}R$; 1.5×10^4cells/well for m$A_{2A}R$) in the absence of G418 20 h prior to assay. CHO-h$A_{2A}R$ cells and CHO-K1 cells transiently expressing m$A_{2A}R$ were washed twice with Hanks' balanced salt solution (HBSS) and incubated in HBSS containing adenosine deaminase (ADA; 3 U/ml) and adenosine 5′ [α,β-methylene] diphosphate (50 μM) for 15

min at 37 °C. Cells were washed twice with HBSS to remove ADA and incubated with rolipram (50 μM), adenosine 5′ [α,β-methylene] diphosphate (50 μM), adenosine, and inosine in the presence or in the absence of dipyridamole at indicated concentration(s) for 10 min at 37 °C. Assay conditions for CHO-hA_{2B}R cells were essentially the same as above with the exception of exclusion of ADA and adenosine 5′ [α,β-methylene] diphosphate pretreatment. Pretreatment was not required for cells expressing the A_{2B}R since no baseline level cAMP signal was detected in the absence of exogenously added adenosine, likely due to the low affinity of the A_{2B}R towards adenosine and the small amount of adenosine produced by the cells during the assay which was not sufficient to activate the A_{2B}R. Intracellular cAMP levels were quantified using an HTRF assay kit (Cisbio).

2.6. Cell-free membrane-based cAMP assay

HEK293-hA_{2A}R cell membranes (PerkinElmer) were incubated in HBSS, containing adenosine 5′ [α,β-methylene] diphosphate (50 μM) and ADA (3 U/ml) at 37 °C for 20 min. Membranes were washed twice with 33mM HEPES containing 0.1 % Tween 20 and stimulated with the same buffer containing 100 μM ATP, 2 μM GTP, 10 μM GDP, 2 μM $MgCl_2$, 150 mM NaCl, 50 μM adenosine 5′ [α,β-methylene] diphosphate, 50 μM rolipram, and inosine (0–300 μM) or CGS 21680 (100 nM) in half-area white plates (Greiner bio-one; 4.5 μg protein/well) for 30 min at 37 °C. cAMP levels were quantified using an HTRF assay kit (Cisbio).

2.7. ERK1/2 phosphorylation assay

CHO-hA_{2A}R cells [23] were seeded in 96-well plates (Greiner bio-one; 2.5×10^4 cells/well) in the absence of G418 20 h prior to assay. Medium was replaced with medium without serum and incubated for an additional 3 h. CHO-hA_{2A}R cells were washed twice with HBSS and incubated in HBSS containing ADA (3 U/ml) and adenosine 5′ [α,β-methylene] diphosphate (50 μM) for 15 min at 37 °C. Cells were washed twice with HBSS to remove ADA and incubated with rolipram (50 μM), adenosine 5′ [α,β-methylene] diphosphate (50 μM), adenosine or inosine at indicated concentration(s) for 20 min at 37 °C. Assay was terminated by aspirating the assay buffer and incubating cells with lysis buffer (50 (μl/well) at room temperature with shaking for 10 min. Phospho ERK1/2 levels were detected using an Alphascreen Surefire kit (PerkinElmer) according the manufacturer suggested protocol. Briefly, 4 μl of the lysate was transferred to a ProxiPlate-384 (PerkinElmer) and incubated with 7 μl of assay detection mixture at room temperature in the dark for 2 h. Fluorescent emissions were quantified using an EnSpire multimode plate reader (PerkinElmer).

2.8. Radioligand displacement assay

The binding of inosine to hA_{2A}R at functional concentrations (cell- and membrane-based cAMP and ERK1/2 phosphorylation) was evaluated using the [^{3}H]-labeled A_{2A}R-selective agonist CGS 21680. HEK293-hA_{2A}R cell membranes (PerkinElmer; 10 μg/well) were pre-incubated in binding buffer (50 mM Tris, 10 mM $MgCl_2$, 1 mM EDTA, 4 U/ml ADA, pH 7.4) in a 96-well MultiScreen plate (Millipore) at 27 °C for 15 min. After addition of varying concentrations of inosine and [^{3}H]CGS 21680 (100 nM), plates were incubated for an additional 75 min followed by washing with cold PBS. Binding was quantified using a MicroBeta TriLux microplate scintillation counter (PerkinElmer). Specific binding was

calculated by subtracting counts obtained in the presence of NECA (50 μM) from total counts.

2.9. Data analysis

Data were analyzed using GraphPad Prism Software. Dose response curves were generated by non-linear regression with a variable slope. Equilibrium binding of [^{3}H]CGS 21680 was analyzed using one-way ANOVA with alpha equal to 0.05. Pooled EC_{50} values for cAMP production and ERK1/2 activation were compared using a paired t test.

3. Results

3.1. Inosine generates an A_{2A}R-selective, inverse agonist-sensitive dynamic mass redistribution (DMR) signal in CHO-hA_{2A}R cells

Signaling through the GPCR family of receptors is complex. Receptor coupling to more than one G protein subunit leading to different signaling outcomes is common. The A_{2A}R couples to two different classes of Gα subunits. A_{2A}R coupling to the Gαs and Gαolf subunits that belong to the Gs class stimulates adenylate cyclase to increase intracellular cAMP upon agonist engagement at the receptor [23], Agonist engagement at the A_{2A}R also leads to ERK1/2 phosphorylation via either cAMP dependent or independent mechanisms [24], However, there are no reports demonstrating that inosine stimulates the A_{2A}R to produce cAMP or ERK1/2 phosphorylation. This may be suggestive of weak stimulation by inosine, hence small differences in cAMP production and ERK1/2 phosphorylation are not readily detectable by the analyte-specific assay technologies utilized. We reasoned that an inosine-mediated, pathway-independent, global and integrated signal would be more detectable than individual, isolated, pathway-specific signals such as cAMP or phosphorylated ERK1/2. Thus we utilized a label-free, biosensor-enabled DMR assay capable of capturing and translating agonist-stimulated signaling events into real-time, quantitative, whole cell phenotypic responses. Applying DMR technology to characterize GPCR signaling in living cells has been well established [25]. Since the human A_{2A}R (hA_{2A}R) is the most pharmacologically characterized A_{2A}R [26] relative to other species, we utilized the hA_{2A}R for interrogating whole cell phenotypic responses emanating from inosine agonism at the A_{2A}R. New knowledge gained here on the hA_{2A}R will serve as a guide to the A_{2A}Rs of other species as this receptor is evolutionarily conserved in terms of sequence, structure and function. In CHO-K1 cells stably transfected with hA_{2A}R (CHO-hA_{2A}R), both adenosine (Fig. 1B) and inosine (Fig. 1C) dose-dependently triggered a whole cell DMR signal that was qualitatively different from that produced by the CHO-K1 cells (Fig. 1A). The A_{2A}R-selective inverse agonist, ZM 241385, inhibited the inosine-induced DMR signal (Fig. 1D) indicating that inosine is capable of generating whole cell phenotypic responses through the A_{2A}R.

3.2. Inosine dose-dependently enhances hA_{2A}R- but not hA_{2B}R-mediated cAMP production

To examine whether inosine-mediated, A_{2A}R-dependent whole cell phenotypic responses are manifestations of increased cAMP production, we evaluated the effects of inosine on cAMP production by CHO-hA_{2A}R. We developed a highly sensitive cell-based cAMP assay by utilizing adenosine deaminase (ADA) to rid the cells of endogenously produced

adenosine and adenosine 5′ [α,β-methylene] diphosphate, an inhibitor of ecto-5′-nucleotidase, to halt endogenous/exogenous production of adenosine and inosine. Using this approach we isolate and decrease background signaling and thereby enhance the signal generated by the addition of inosine. In this assay, adenosine increased cAMP production with an EC_{50} (half maximal effective concentration) of 6 ± 1 nM and an E_{max} (maximal stimulation) of 48 nM (Fig. 2A; Table 1). Similarly, inosine dose-dependently increased cAMP production with an EC_{50} of 300.7 ± 48.9 μM (Table 1) and E_{max} of 80 nM (Fig. 2B; Table 1). However, EC_{50} values for adenosine and inosine were significantly different ($p < 0.0001$). To rule out the unlikely possibility that $hA_{2A}R$ stimulation was due to the presence of adenosine as an impurity, we analyzed an 800 μM inosine stock solution by HPLC and found no trace of adenosine (≤ 0.025%). The inosine-induced increase in cAMP production was completely inhibitable by the $A_{2A}R$ inverse agonist ZM 241385, demonstrating the specificity of the inosine-$hA_{2A}R$ interaction (Fig. 2B; Table 1). As expected, ZM 241385 also inhibited adenosine-induced cAMP production however, the inhibition was partial (Fig 2A; Table 1; EC_{50}=2.45 ± 1.29 μM). These results suggest a potency and/or a pharmacological difference between inosine and adenosine at the $A_{2A}R$, and rule out the remote possibility of $hA_{2A}R$ stimulation by undetectable amounts of adenosine that may be present in inosine.

The $A_{2B}R$ also couples to Gαs, hence it stimulates cAMP production upon activation. Therefore, we addressed whether inosine increased cAMP production by CHO-K1 cells stably expressing the human $A_{2B}R$ (CHO-$hA_{2B}R$). Although adenosine increased cAMP production by CHO-$hA_{2B}R$ cells, inosine did not, even at concentrations equal to and above those that stimulated cAMP production by CHO-$hA_{2A}R$ bearing cells (Fig. 2C). These results demonstrate that inosine engagement at the $A_{2A}R$, but not at the $A_{2B}R$, leads to stimulation of adenylate cyclase and a rise in intracellular cAMP levels.

To examine the notion that inosine actions at the $A_{2A}R$ are indirect and that inosine influences the extracellular concentration of adenosine through equilibrative nucleoside transporter 1 (ENT1) and ENT2 to activate the $A_{2A}R$, we utilized saturating concentrations of an ENT1 and ENT2 inhibitor, dipyridamole. Inhibition of ENT1 and ENT2 with dipyridamole slightly reduced the baseline while slightly increasing the maximal cAMP production in response to adenosine (Fig. 3A) indicating that this cell-based assay a) is minimally influenced by endogenously produced adenosine and b) faithfully reports $A_{2A}R$ activation by the exogenous agonist. Similarly, dipyridamole marginally reduced baseline cAMP levels while enhancing maximum inosine-mediated cAMP production (Fig. 3B) demonstrating that inosine directly and dose-dependently activates the $A_{2A}R$.

We also evaluated the effects of metabolites of inosine on $A_{2A}R$ function. As shown in Fig. 4, inosine monophosphate is approximately 20-fold less potent than inosine in activating the $A_{2A}R$. We also observed that xanthine is more potent than inosine monophosphate whereas hypoxanthine is equipotent to inosine monophosphate at the $A_{2A}R$ (Fig. 4) indicating that inosine metabolites are poor agonists relative to adenosine (EC_{50}=0.006 ± 0.001 μM) and inosine (EC_{50}= 300.7 ± 48.9 μM) at the receptor.

3.3. Inosine dose-dependently increases mouse $A_{2A}R$ ($mA_{2A}R$)-mediated intracellular cAMP production

In mouse models of human disease, the role of the $A_{2A}R$ in inosine-mediated inhibition of inflammation [12-15] and pain [27] has been convincingly demonstrated utilizing gene ablation and pharmacological tools. As inosine can be readily converted to adenosine in vivo, it is not clear whether the in vivo therapeutic effects of inosine mediated via the $A_{2A}R$ are direct or indirect in nature. We therefore investigated the ability of inosine to activate the $mA_{2A}R$ in CHO-K1 cells transiently expressing the receptor to produce cAMP. CHO-K1 cells were chosen as the host cells as they do not have endogenous expression of any adenosine receptor subtype [23]. Both adenosine and the $A_{2A}R$ -selective agonist CGS 21680 increased the intracellular production of cAMP by CHO-K1 cells transiently expressing $mA_{2A}R$, while the $A_{2A}R$-selective inverse agonist ZM 241385 inhibited it (Fig. 5A). In contrast, vector-transfected CHO-K1 cells did not yield measurable cAMP levels under any condition. As seen with adenosine, inosine dose-dependently increased cAMP production (Fig. 5A). Moreover, dose response analyses indicated that the EC_{50} values of adenosine and inosine were 0.059 ± 0.02 μM and 709 ± 135.2 μM, respectively (Fig. 5B and 5C; Table 1). Relative to adenosine, inosine-induced cAMP production appeared to be more sensitive to the $A_{2A}R$ inverse agonist ZM 241385. Taken together, these results suggest that inosine, although not as potent as adenosine, is a functional agonist at the $mA_{2A}R$.

3.4. Inosine dose-dependently induces $hA_{2A}R$-mediated ERK1/2 activation

Agonist engagement at the $A_{2A}R$ leads to ERK1/2 phosphorylation and subsequent activation [28,29]. Depending on the cell type, the signaling pathways leading to $A_{2A}R$-mediated ERK1/2 activation can vary. Gαs-dependent ERK1/2 activation is reported in CHO-K1 cells, whereas Gs-independent ERK1/2 activation is reported in HEK293 cells [28]. Analysis of receptor truncations indicates that Gαs engagement/cAMP production and ERK1/2 phosphorylation/activation are functionally separable [30]. To investigate whether inosine agonism at the $A_{2A}R$ results in ERK1/2 activation, we evaluated the effects of inosine on ERK1/2 phosphorylation in CHO-$hA_{2A}R$ cells. As shown in Fig. 6A and Table 1, adenosine stimulated ERK1/2 phosphorylation with an EC_{50} of 0.211 ± 0.036 μM. Similarly, inosine dose-dependently enhanced ERK1/2 phosphorylation with an EC_{50} of 89.38 ± 15.12 μM (Fig. 6B; Table 1). Both adenosine- and inosine-induced ERK1/2 phosphorylation were inhibited by the $A_{2A}R$ inverse agonist ZM 241385 (Fig. 6A & Fig. 6B; Table 1). Comparison of EC_{50} values of adenosine indicated a 35-fold preference for cAMP production than for ERK1/2 phosphorylation ($p < 0.001$). In contrast, inosine exhibited a 3.3-fold preference for ERK1/2 phosphorylation over cAMP production ($p < 0.005$). These results further demonstrate that inosine is an agonist at the $A_{2A}R$ and that the inosine-engaged receptor has a different pharmacological profile relative to the adenosine-engaged receptor.

3.5. Inosine dose-dependently stimulates $hA_{2A}R$-mediated cAMP production in a cell-free, membrane-based system

In the cell-based $A_{2A}R$ functional assay, one can conceptualize a scenario where the exogenously added inosine is taken up, converted to adenosine intracellularly and exported

back out to engage and activate the receptor. Such a scenario is highly unlikely given the time scale (10 min) of the cell-based assay and maintenance of a cellular response in the presence of the ENT1 and ENT2 inhibitor dipyridamole in intact cell assays. However, to further rule out the possibility, we evaluated the ability of inosine to activate the $A_{2A}R$ in HEK293-h$A_{2A}R$ cytosol-free membrane preparations. To this end, we established a sensitive, cell-free, membrane-based assay for the h$A_{2A}R$. The $A_{2A}R$-selective agonist CGS 21680 increased cAMP production, while the $A_{2A}R$-selective inverse agonist ZM 241385 reduced cAMP production to levels that were not detectable by the assay, demonstrating that this assay reports bona fide $A_{2A}R$ receptor pharmacology (Fig. 7A). Inosine dose-dependently stimulated cAMP production while ZM 241385 inhibited inosine-induced cAMP production in this cell-free, membrane-based assay (Fig. 7B), indicating that the inosine effects at the $A_{2A}R$ are direct, and thus providing further evidence for inosine agonism at this receptor.

3.6. Inosine displaces the h$A_{2A}R$-selective agonist, CGS 21680, binding to the receptor

In general, agonist-mediated GPCR stimulation stems from agonist binding at the receptor that is detectable by saturation binding experiments. On the basis of our observations of inosine-mediated h$A_{2A}R$ activation and subsequent generation of cAMP and ERK1/2 responses in the cell-based assays, we hypothesized that inosine may bind to the h$A_{2A}R$ with high enough affinity to be detectable in an equilibrium binding assay. As there are no commercially available radiolabeled inosine analogs to evaluate direct binding, we determined whether inosine could displace binding of the high affinity h$A_{2A}R$ agonist, CGS 21680. In equilibrium binding assays, the specific binding of [^{3}H]CGS 21680 to the $A_{2A}R$ was reduced by approximately 20% regardless of the inosine concentration ($F [4, 73] = 7.287$; $P<0.0001$) in the presence of inosine (Fig. 8) supporting the notion that inosine competes with the high affinity adenosine analog CGS 21680 for the same binding site at the $A_{2A}R$. The low-magnitude displacement of the [^{3}H]CGS 21680 is consistent with inosine being a low affinity agonist.

4. Discussion

Inosine is a ubiquitous purine nucleoside that exerts anti-inflammatory and immunomodulatory effects. Inosine and metabolically stable analogs of inosine [31,32] utilize adenosine receptors to modulate anti-inflammatory effects. There are four adenosine receptor (AR) subtypes termed A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R. Among them, $A_{2A}R$ is the most effective receptor in downregulating inflammation [33,34]. The role of the $A_{2A}R$, however, is less clear in the context of inosine-mediated anti-inflammation and immunomodulation. There is a discrepancy between postulating inosine-mediated in vivo anti-inflammatory effects requiring the $A_{2A}R$ [20,21] when in vitro studies report no functional activation of the $A_{2A}R$ [22] or displacement of a high-affinity agonist bound to the $A_{2A}R$ in the presence of inosine [7], Here we examined the possibility of inosine-mediated $A_{2A}R$ activation in vitro using a variety of highly sensitive and selective experimental approaches not applied previously to the evaluation of inosine receptor activation.

Our investigations produced several lines of experimental evidence supporting inosine as a specific $A_{2A}R$ agonist. First, in CHO-K1 cells that do not express any endogenous adenosine receptor subtypes [23], inosine dose-dependently increased cAMP production upon exogenous expression of both mouse ($mA_{2A}R$) and human $A_{2A}Rs$ ($hA_{2A}R$). This inosine-induced cAMP production was inhibited by the $A_{2A}R$-selective inverse agonist ZM 241385. Second, inosine-induced cAMP production by CHO-$hA_{2A}R$ cells was not altered in the presence of the ENT1 and 2 inhibitor dipyridamole. Third, in CHO-$hA_{2A}R$ cells, inosine exhibited a dose-dependent enhancement of ERK1/2 phosphorylation that was sensitive to ZM 241385. Fourth, consistent with $A_{2A}R$ activation in the cell-based assays, inosine exhibited a ZM 241385-sensitive induction of cAMP production in cell-free membrane preparations containing the $hA_{2A}R$. This further supports the notion that it is inosine, and not a cellular metabolic byproduct of inosine, that is responsible for the stimulation of the $A_{2A}R$. Finally, in equilibrium binding assays, inosine inhibited the binding of the $A_{2A}R$-selective agonist CGS 21680 to CHO-$hA_{2A}R$ membranes.

Our data indicate that inosine is a less potent agonist than adenosine at the $A_{2A}R$. In cell-based cAMP assays, inosine concentrations that activate the $mA_{2A}R$ and the $hA_{2A}R$ are in the high micromolar range, hence generation of accurate EC_{50} values are not possible. The approximate EC_{50} values of inosine for cAMP production for the $hA_{2A}R$ and $mA_{2A}R$ s are 300.7 ± 48.9 μM and 709 ± 135.2 μM, respectively, indicating that, relative to adenosine, inosine is approximately four orders of magnitude less potent at the $A_{2A}R$. In ERK1/2 phosphorylation assays, EC_{50} values for adenosine and inosine are 0.211 ± 0.036 μM and 89.38 ± 15.12 μM, respectively, a potency difference of 423-fold. Although the EC_{50} values of inosine appear relatively high, they are well within the physiological levels achieved in disease settings [7,8].

The low potency of inosine relative to adenosine at the A_2aR may be explained by the nature of its interactions with the critical residues that constitute the agonist-binding pocket of the receptor. Crystal structure predictions in combination with site-directed mutagenesis indicate that Asn^{253} in the sixth transmembrane domain of the $hA_{2A}R$ is the critical residue that defines the hydrophobic pocket in which the adenine ring of the agonist rests [35]. In the case of adenosine, two hydrogen bond interactions of Asn^{253} with N^6 and N^7 of adenine (Fig. 9; [36]) stabilize adenosine binding at the $A_{2A}R$. Inosine, a metabolite of adenosine, differs from adenosine by having only N^7 but not N^6 in the adenine ring, and thus is capable of having only one hydrogen bond interaction with Asn^{253} in the agonist-binding pocket. This may explain at least in part the observed low affinity interaction between inosine and the receptor relative to that of adenosine as evident from the agonist displacement and functional assays.

We have found that adenosine and inosine possess uniquely different pharmacological profiles. The EC_{50} values for cAMP production and ERK1/2 activation reveal that both adenosine and inosine exhibit an agonist-specific signaling bias. Adenosine is 35-fold more potent in stimulating $hA_{2A}R$-mediated cAMP production relative to $hA_{2A}R$-mediated ERK1/2 phosphorylation, thus adenosine is a cAMP-biased agonist. In contrast, inosine is 3.3-fold more potent in stimulating $hA_{2A}R$-mediated ERK1/2 phosphorylation than $hA_{2A}R$-mediated cAMP production, hence it exhibits an ERK1/2 signaling bias. Additionally,

inosine-stimulated ERK1/2 phosphorylation is sensitive to the $A_{2A}R$ reverse agonist ZM 241385 to a greater extent than adenosine-stimulated ERK1/2 phosphorylation. These findings suggest that adenosine and its primary metabolite, inosine, are uniquely different agonists with signaling biases capable of producing overlapping as well as distinct pharmacological outcomes.

Using an equilibrium competitive binding assay, we demonstrate that inosine displaces the binding of the $A_{2A}R$-selective agonist CGS 21689 to the $hA_{2A}R$. This observation differs, however, from a previous report [7] wherein inosine failed to displace binding of the A_1R/A_3R agonist N6-(3-iodo-4-aminobenzyl)adenosine [37,38] to the rat $A_{2A}R$. Differences in species and in affinity of the agonist utilized in these studies may explain in part the differing outcomes.

Adenosine activates both the $A_{2A}R$ and $A_{2B}R$. The $A_{2A}R$ is the high affinity receptor that is coupled to Gs and Golf and plays a functionally non-redundant role in down modulating inflammation. The $A_{2B}R$ is the low affinity receptor that is coupled to Gs and Gq, shows Gs-dependent ERK1/2 activation [24] and produces both pro- and anti-inflammatory effects [39], The anti-inflammatory and immunomodulatory roles of both $A_{2A}R$ and $A_{2B}Rs$ have been largely attributed to Gs-dependent production of cAMP [33,34], Although the significance of $A_{2A}R$-mediated ERK1/2 activation in the context of inflammation and immunomodulation is largely unknown, $A_{2A}R$-mediated ERK1/2 activation promotes cell death [40], cell proliferation [40] and inhibits osteoclast formation [41]. Our finding that inosine, the primary metabolite of adenosine, is a signaling-biased agonist at the $A_{2A}R$ but not at the $A_{2B}R$ suggests a wide variety of functional outcomes that are dependent upon adenosine and inosine levels and relative receptor abundance in vivo.

The major outcome of our investigation is the identification of inosine, the first metabolite of the breakdown of adenosine, as a functional agonist of the $A_{2A}R$. The demonstration of inosine as a functional agonist at the $A_{2A}R$ increases our understanding of $A_{2A}R$ signaling with respect to the regulation of inflammation and immunomodulation. The current paradigm of $A_{2A}R$ is based on the notion that the endogenous immunomodulator, adenosine, is the sole natural agonist of the $A_{2A}R$. Adenosine, having a half-life <10 s in vivo [1], is rapidly deaminated to inosine, rendering it inactive at the $A_{2A}R$ [22]. Hence, adenosine-mediated $A_{2A}R$ signaling may be short-lived. Identifying inosine as an agonist at the $A_{2A}R$ expands upon this concept and supports the notion that deamination does not render adenosine completely inactive at the $A_{2A}R$, but rather produces an agonist, inosine, with a relatively longer half-life (15 h; [2]) and elevated interstitial levels that may prolong $A_{2A}R$ signaling to sustain anti-inflammatory and immunomodulatory responses. We envision that these two endogenous modulators acting together produce prolonged and accentuated activation of the $A_{2A}R$, an effect that neither one can achieve individually. Adenosine engages the $A_{2A}R$ to generate initial rapid and robust cAMP-biased responses that are subsequently shifted towards ERK1/2 biased responses by inosine. Such dual agonist-mediated signaling may be crucial for mounting a more robust and effective immunomodulatory response in order to limit the extent of tissue damage in inflammatory diseases. Adenosine-driven immunomodulation may be more focused at the disease site where adenosine is produced, released and degraded. In contrast, inosine, being relatively

more stable, accumulates, functions locally and due to its stability may diffuse more broadly. Understanding the pharmacological intricacies of interplay between these two natural anti-inflammatory and immunomodulatory agonists and the $A_{2A}R$ may provide new insights into potential therapeutic interventions of inflammation and immune-mediated diseases. Additionally, quantification of the relative potency and efficacy of inosine and adenosine at the $A_{2A}R$ will aid our understanding of agonist-receptor interactions at the binding pocket that in turn may lead to the design of agonists with beneficial pharmacological profiles.

Acknowledgements

We gratefully acknowledge Dr. Karl-Norbert Klotz for providing CHO-hA_{2A}R and CHO-hA_{2B}R cells and Dr. Jan Rydzewski for HPLC analysis. This work was supported in part by NIH grant 5R21AI105518 from the National Institute of Allergy and Infectious Diseases.

Research reported in this publication was supported in part by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under Award Number R21AI105518. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Abbreviations

ADA	adenosine deaminase
Ado	adenosine
AR	adenosine receptor
A_1R	adenosine A_1 receptor
$A_{2A}R$	adenosine A_{2A} receptor
$A_{2B}R$	adenosine A_{2A} receptor
A_3R	adenosine A_3 receptor
cAMP	cyclic adenosine monophosphate
CGS 21680	4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl] benzenepropanoic acid hydrochloride
DMPX	3,7-Dimethyl-l-propargylxanthine
DMSO	dimethyl sulfoxide
DMR	dynamic mass redistribution
ENT1	equilibrative nucleoside transporter 1
ENT2	equilibrative nucleoside transporter 2
ERK1/2	extracellular signal-regulated kinase-1 and -2
GPCR	G protein-coupled receptor
HBSS	Hanks' balanced salt solution
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid

HTRF	homogeneous time resolved fluorescence
Ino	inosine
LPS	lipopolysaccharide
NECA	5′-N-Ethylcarboxamidoadenosine
PBS	phosphate buffered saline
SD	standard deviation
SEM	standard error of the mean
TNBS	2,4,6-trinitrobenzenesulfonic acid
ZM 241385	4-(2-[7-Amino-2-(2-furyl)[l,2,4]triazolo[2,3-a][l,3,5]triazin-5-ylamino]ethyl)phenol

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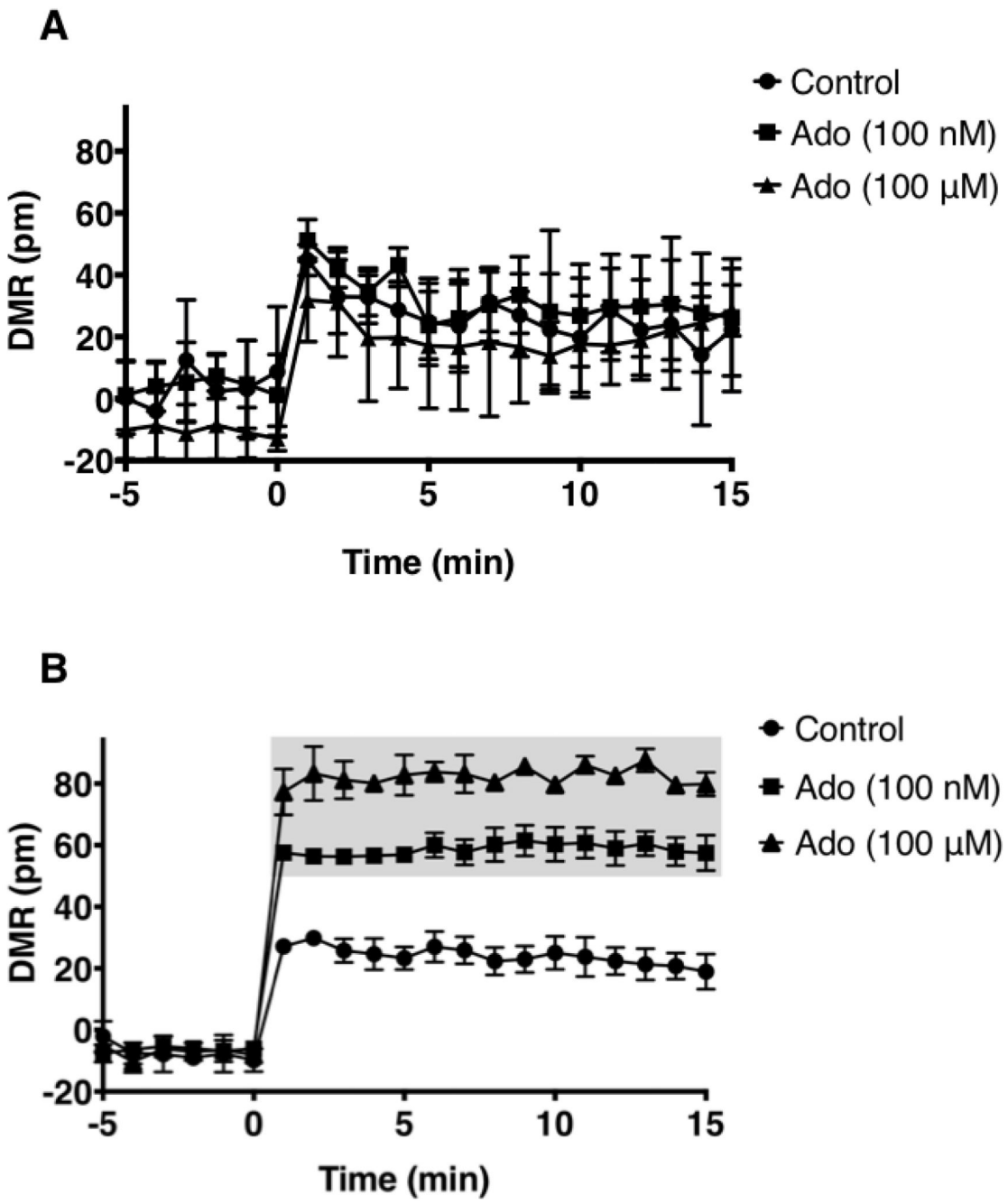
Highlights

- Inosine directly and physically engages the $A_{2A}R$.
- Inosine dose-dependently stimulates cAMP production mediated through the $A_{2A}R$.
- Inosine dose-dependently induces $A_{2A}R$-mediated ERK1/2 phosphorylation.
- Adenosine and inosine differ in their signaling biases at the $A_{2A}R$.



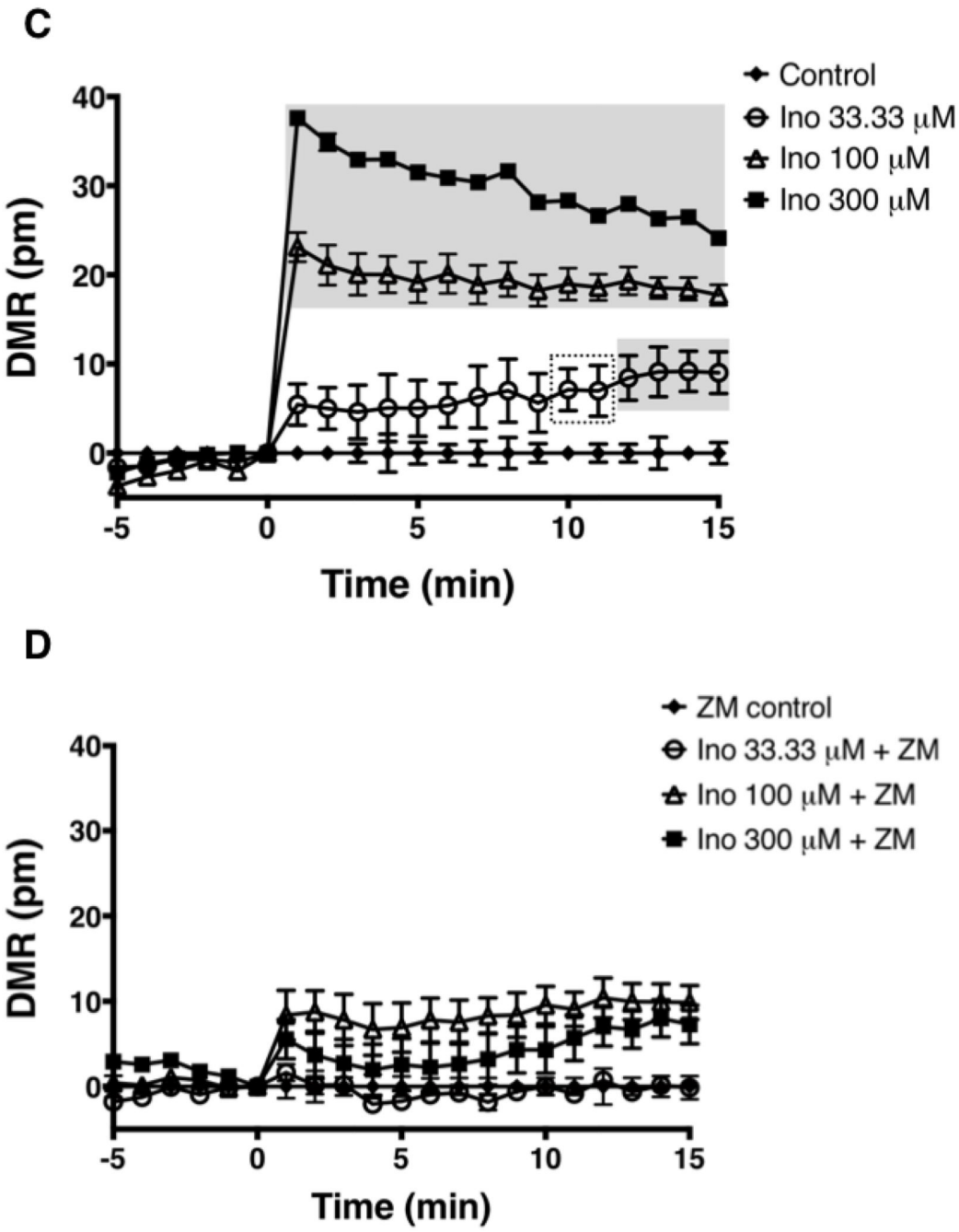
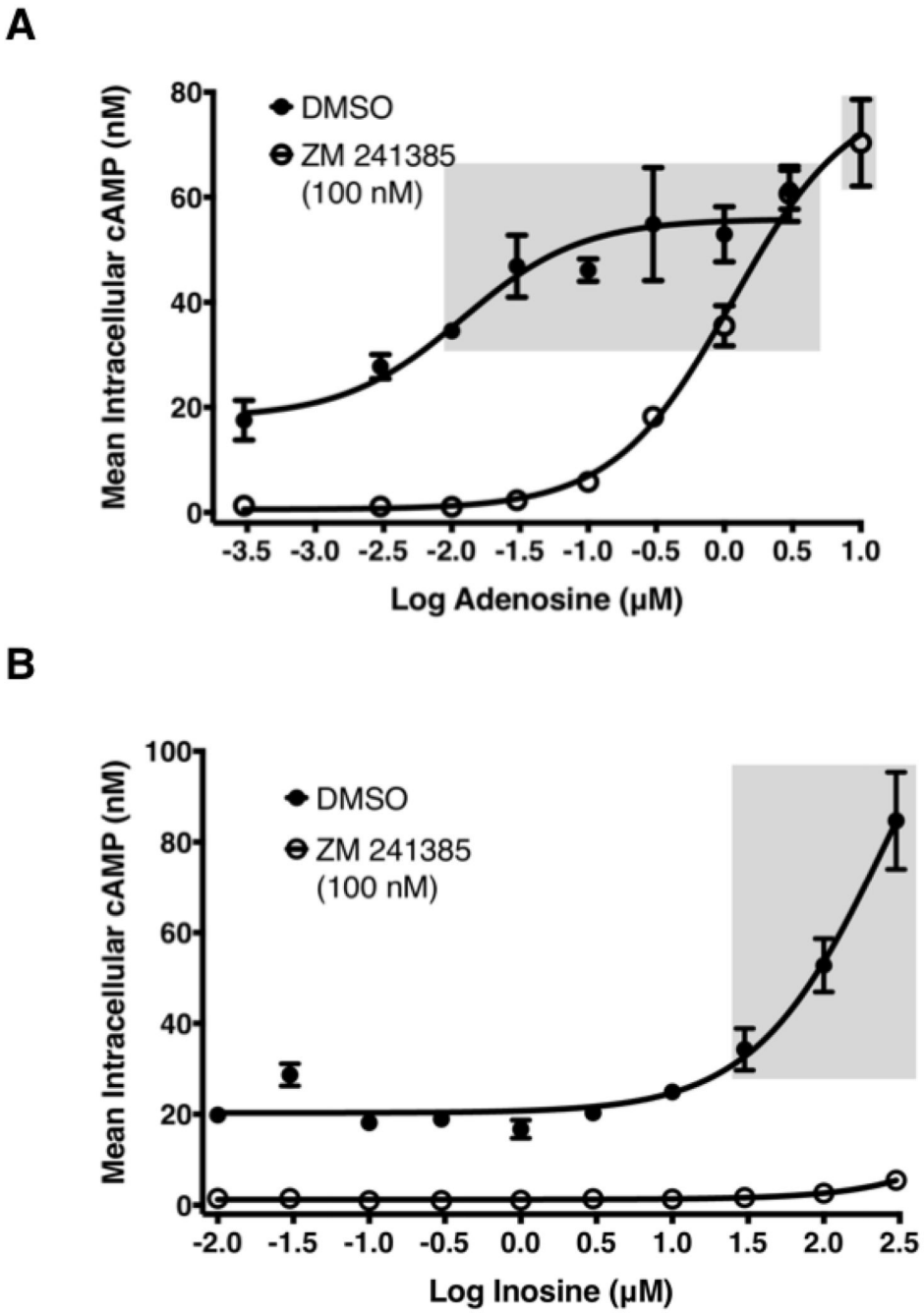


Fig. 1.

Dynamic mass redistribution analysis of ARs on CHO-K1 and CHO-hA_{2A}R cells. DMR response traces of CHO-K1 (A) and CHO-hA_{2A}R (B, C & D) stimulated with indicated concentrations of adenosine (Ado; A & B), inosine (Ino; C & D) and 100 nM ZM 241385 (ZM; D). All data are mean values ± SEM of a representative independent experiment (n=3). Data with $p < 0.001$ and $p < 0.05$ relative to control are indicated in shaded and dotted boxes, respectively.



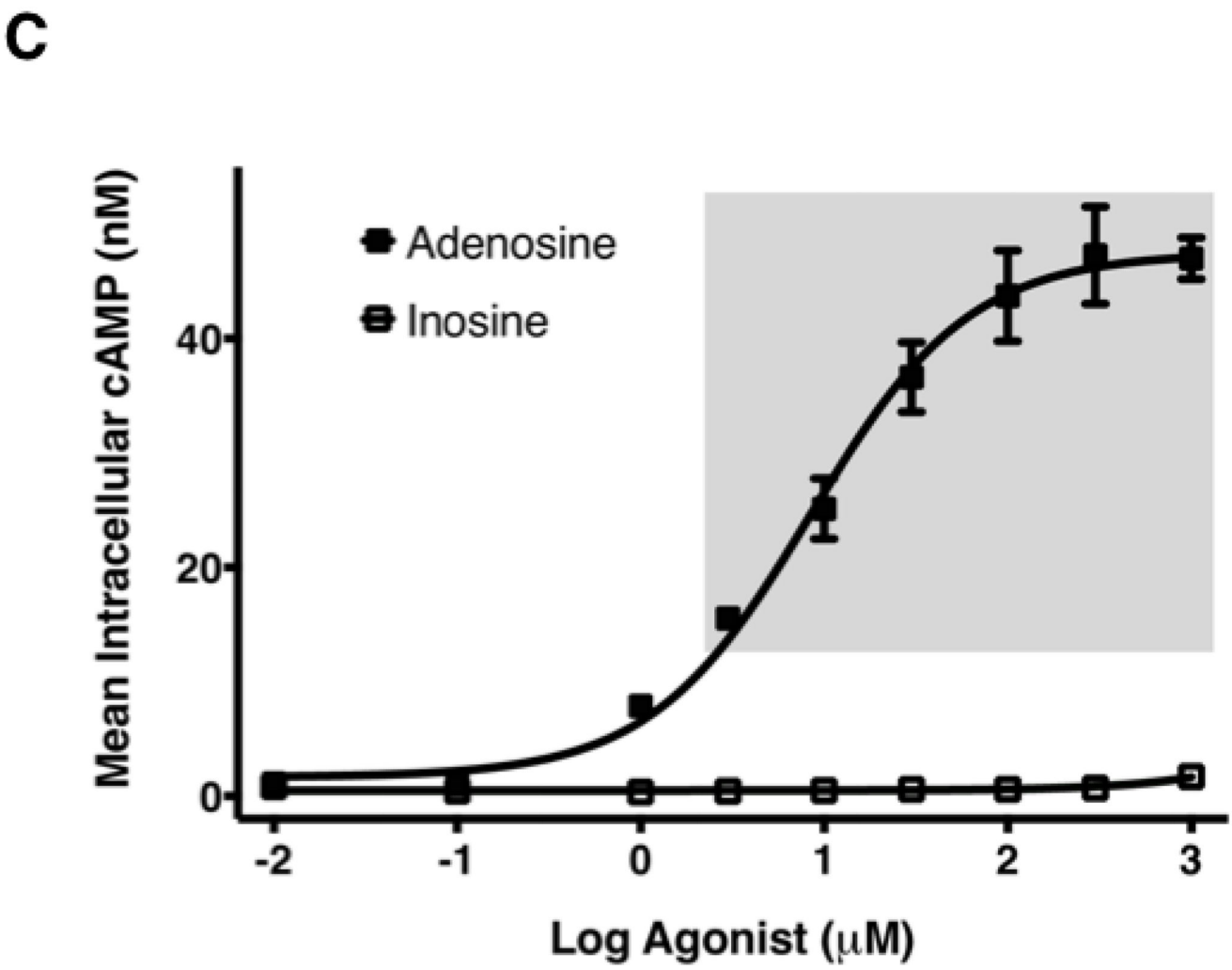


Fig. 2.

Effect of adenosine and inosine on $hA_{2A}R$- and $hA_{2B}R$-mediated intracellular cAMP production.

Adenosine stimulated $hA_{2A}R$-mediated (A) and $hA_{2B}R$-mediated (C) cAMP production, while inosine stimulated $hA_{2A}R$-mediated (B) but not $hA_{2B}R$-mediated (C) cAMP production. CHO-$hA_{2A}R$ (A and B) and CHO-$hA_{2B}R$ (C) cells were incubated with adenosine (A and C) and inosine (B and C) in the presence or in the absence of the $hA_{2A}R$-selective inverse agonist ZM 241385 (A and B) for 10 min. Mean intracellular cAMP levels ± SEM of a representative experiment are shown (n=3). Shaded boxes indicate data with $p < 0.001$ relative to control. EC_{50} values with 95% confidence intervals (in parentheses) were 12(4–33) nM and 259.9(104–651) μM for adenosine and inosine respectively.

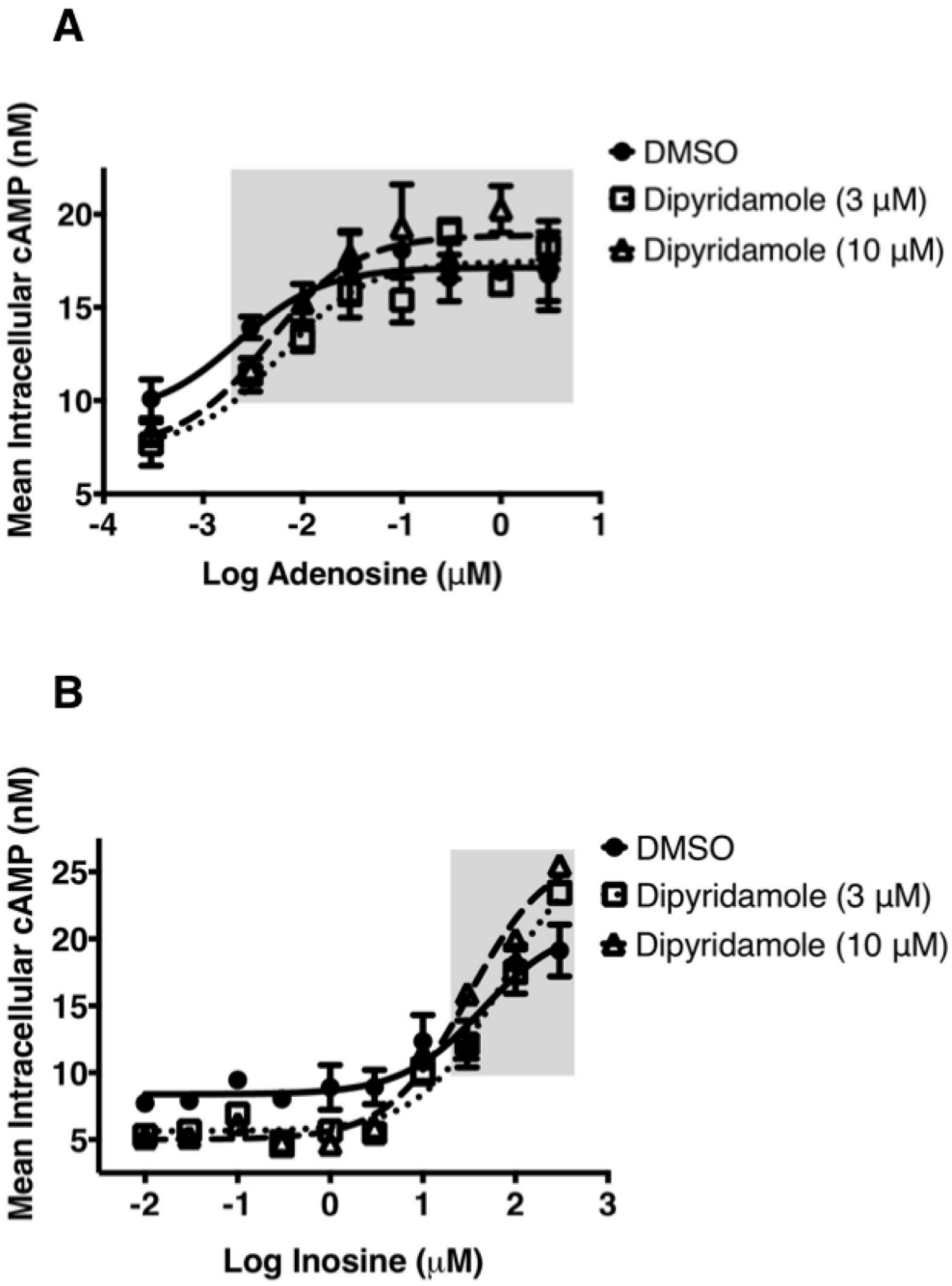


Fig. 3.

Effect of ENT1/ENT2 inhibition on $hA_{2A}R$-mediated intracellular cAMP production.

The ENT1 and ENT2 inhibitor dipyridamole marginally reduces baseline cAMP production and marginally enhances adenosine- and inosine-mediated cAMP production. CHO-$hA_{2A}R$ cells were incubated with adenosine (A) and inosine (B) in the presence or in the absence of the indicated concentrations of dipyridamole for 10 min. Mean intracellular cAMP levels ± SEM of a representative experiment are shown (n=4). Shaded boxes indicate data with $p < 0.001$ relative to control.

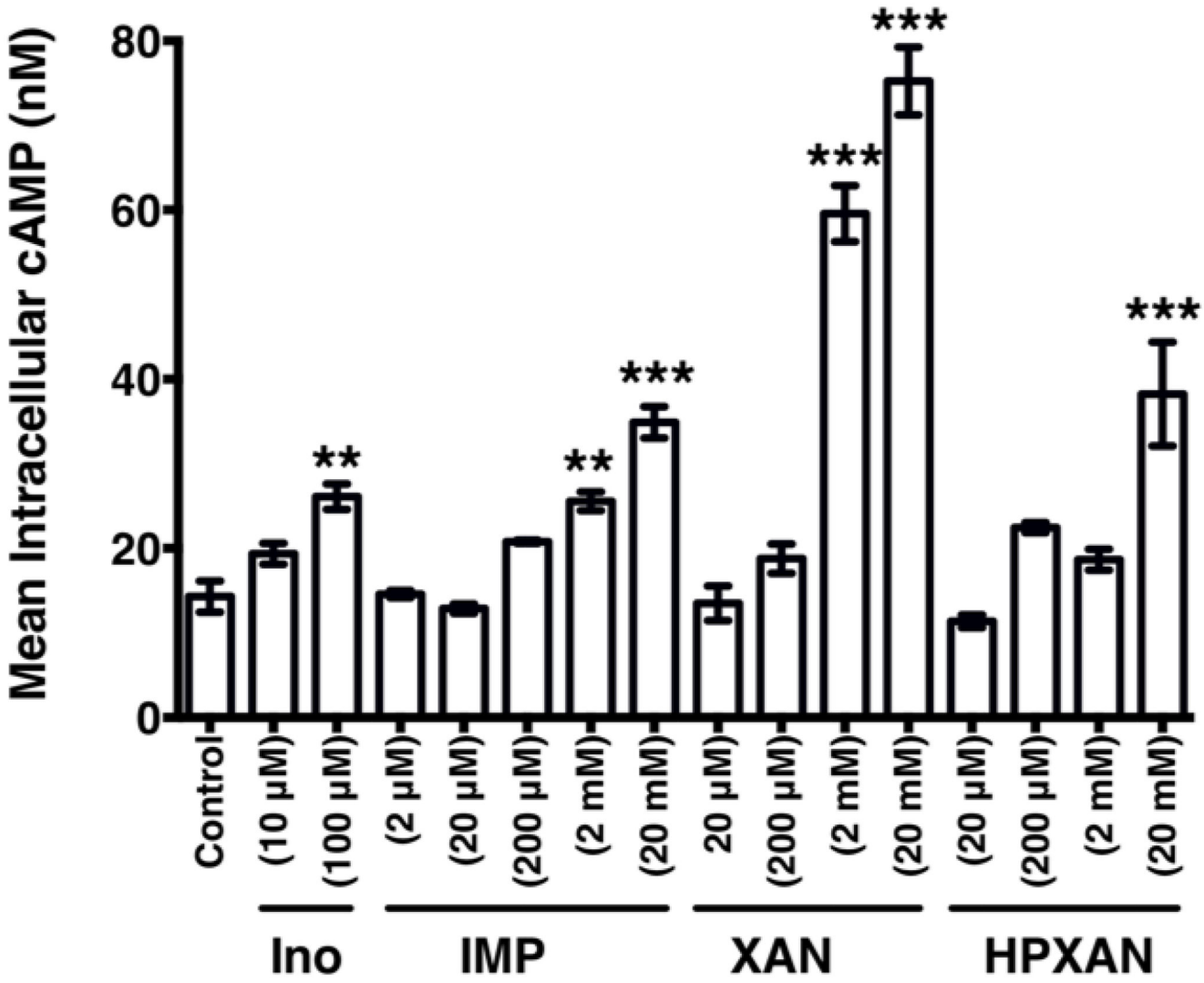
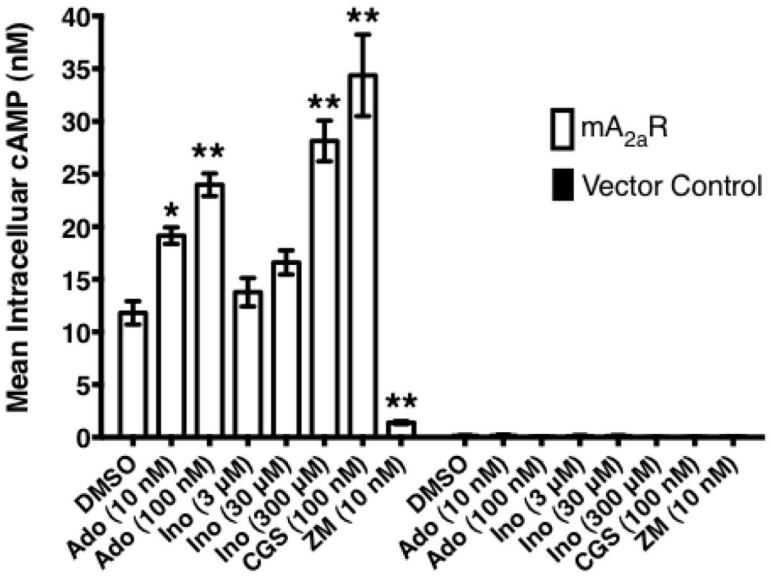


Fig. 4.

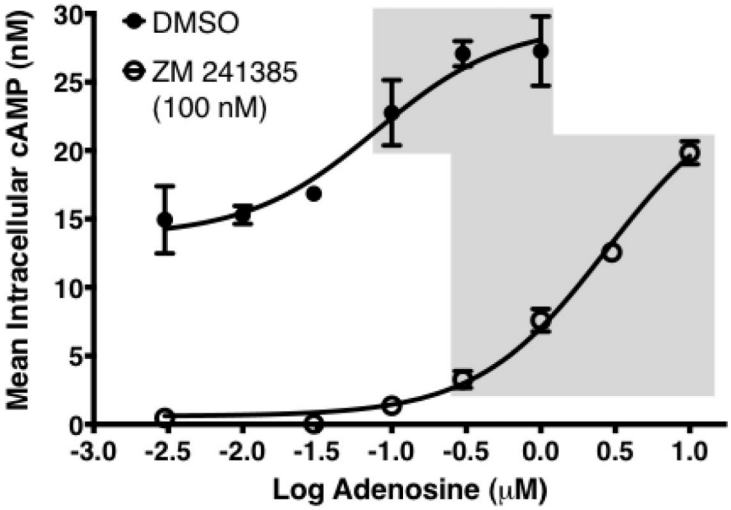
Metabolites of inosine are less potent in inducing $hA_{2A}R$-mediated intracellular cAMP production than inosine.

CHO-$hA_{2A}R$ cells were incubated with the indicated concentrations of inosine (Ino), inosine monophosphate (IMP), xanthine (XAN) and hypoxanthine (HPXAN). Mean intracellular cAMP levels ± SEM of a representative experiment are shown (n=3; **, $P < 0.01$; *** $p < 0.001$ vs control).

A



B



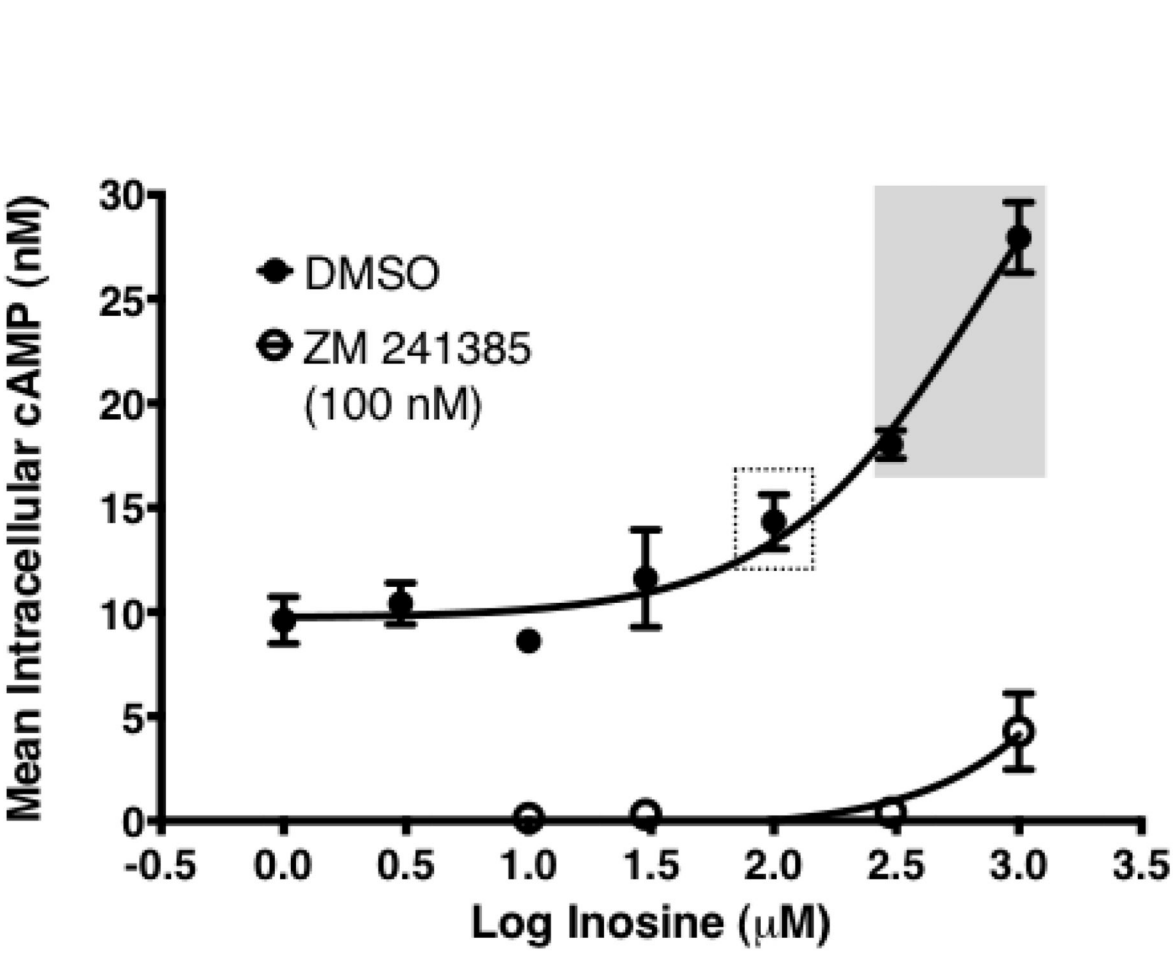


Fig. 5.

Inosine induces $mA_{2A}R$-mediated intracellular cAMP production.

(A) CHO-K1 cells transiently transfected with $mA_{2A}R$ but not with vector alone exhibit agonist-inducible cAMP production. Transiently transfected CHO-K1 cells were incubated with various agonists and an inverse agonist for 10 min. Mean intracellular cAMP levels ± SEM of representative experiments are shown (n=3; *, $p < 0.05$; **, $p < 0.01$ vs DMSO). Adenosine (Ado), inosine (Ino), $A_{2A}R$-selective agonist CGS 21680 (CGS) and $A_{2A}R$-selective inverse agonist ZM 241385 (ZM). Dose response of adenosine (B) and inosine (C) to $mA_{2A}R$. CHO-K1 cells transiently transfected with $mA_{2A}R$ were incubated with various concentrations of adenosine and inosine. Mean cAMP levels ± SEM of a representative experiment in triplicates are shown. Shaded and dotted boxes indicate data with $p < 0.001$ and $p < 0.05$ relative to control, respectively. EC_{50} values with 95% confidence intervals (in parentheses) were 79(21.5–293) nM and 773(249.5–2396) μM for adenosine and inosine respectively.

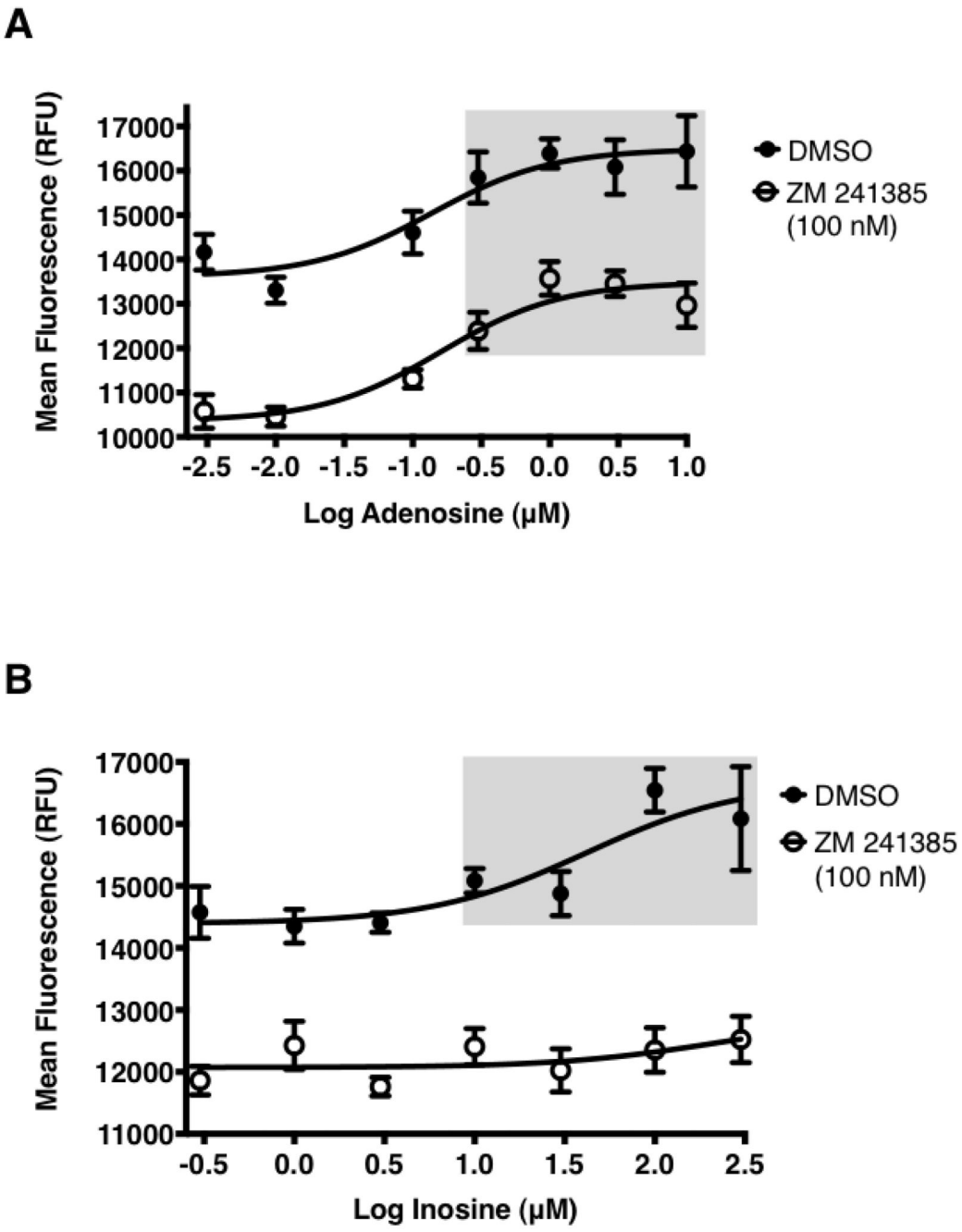
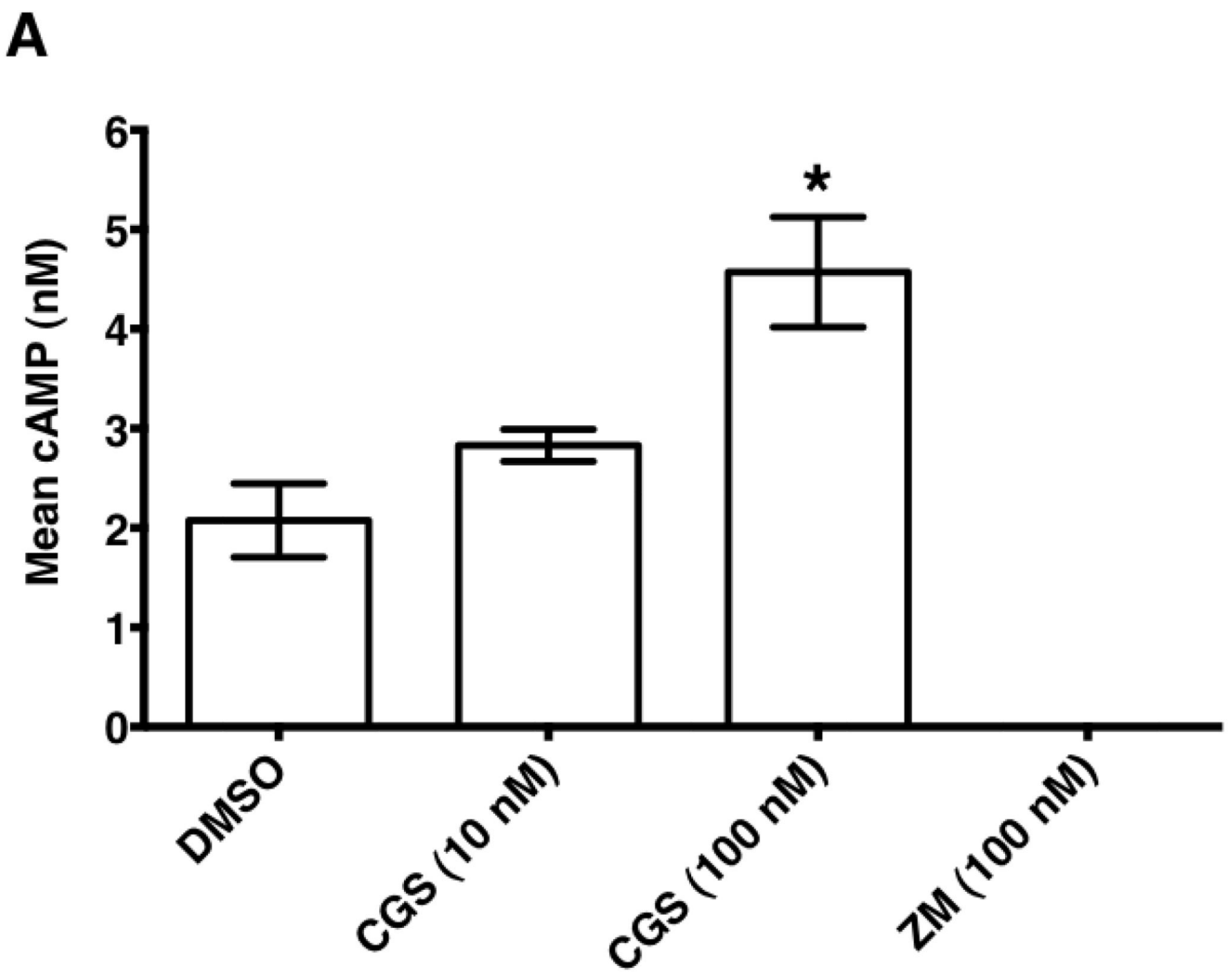


Fig. 6.

Both adenosine and inosine dose-dependently induce $hA_{2A}R$-mediated ERK1/2 phosphorylation.

Adenosine- (A) and inosine- (B) stimulated ERK1/2 phosphorylation by CHO-$hA_{2A}R$ cells. CHO-$hA_{2A}R$ cells were incubated with various concentrations of adenosine or inosine for 20 min. Phospho ERK1/2 levels were quantified using a FRET-based detection kit. Mean FRET ratios ± SEM of representative experiments are shown (n=3). Shaded boxes indicate data with $p < 0.001$ relative to control. EC_{50} values with 95% confidence intervals (in

parentheses) were 139 (28–680) nM and 41.9 (5.8–303) μM for adenosine and inosine respectively. The A_{2A}R-selective inverse agonist ZM 241385 (ZM) blocked adenosine- and inosine-stimulated ERK1/2 phosphorylation.



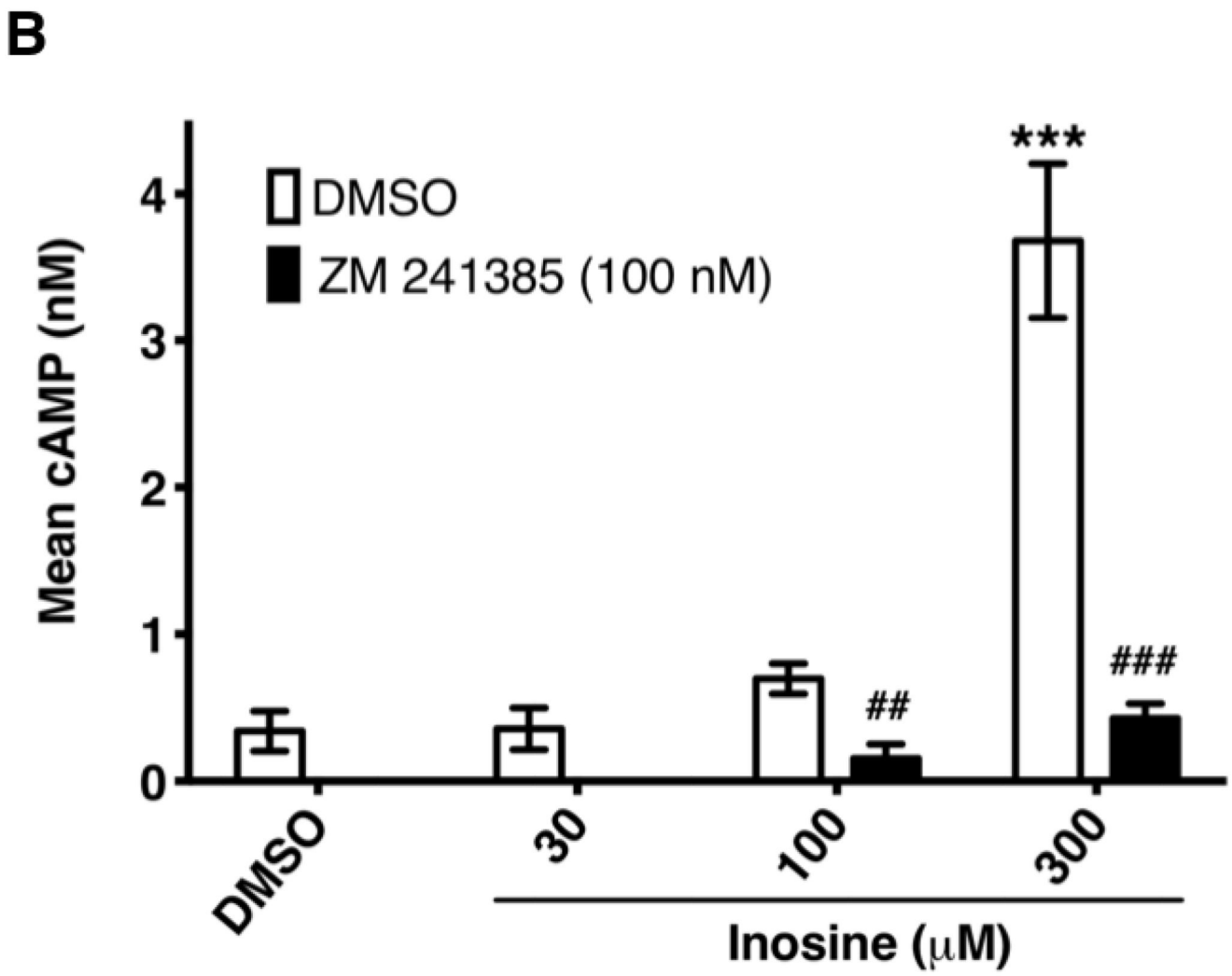


Fig. 7.

Inosine dose-dependently induces $hA_{2A}R$-mediated cAMP production in cell-free membrane preparations.

CHO-$hA_{2A}R$ cell membranes were incubated with indicated concentrations of CGS 21680 (CGS) and ZM 241385 (ZM) (A) and with inosine (B) in the presence and in the absence of the $A_{2A}R$-selective inverse agonist ZM 241385 for 30 min. Mean cAMP production ± SEM of representative experiments are shown (n=6; * and ***, $p < 0.05$ and $p < 0.001$ vs DMSO respectively; ## and ###, $p < 0.001$ and $p < 0.001$ vs inosine respectively).

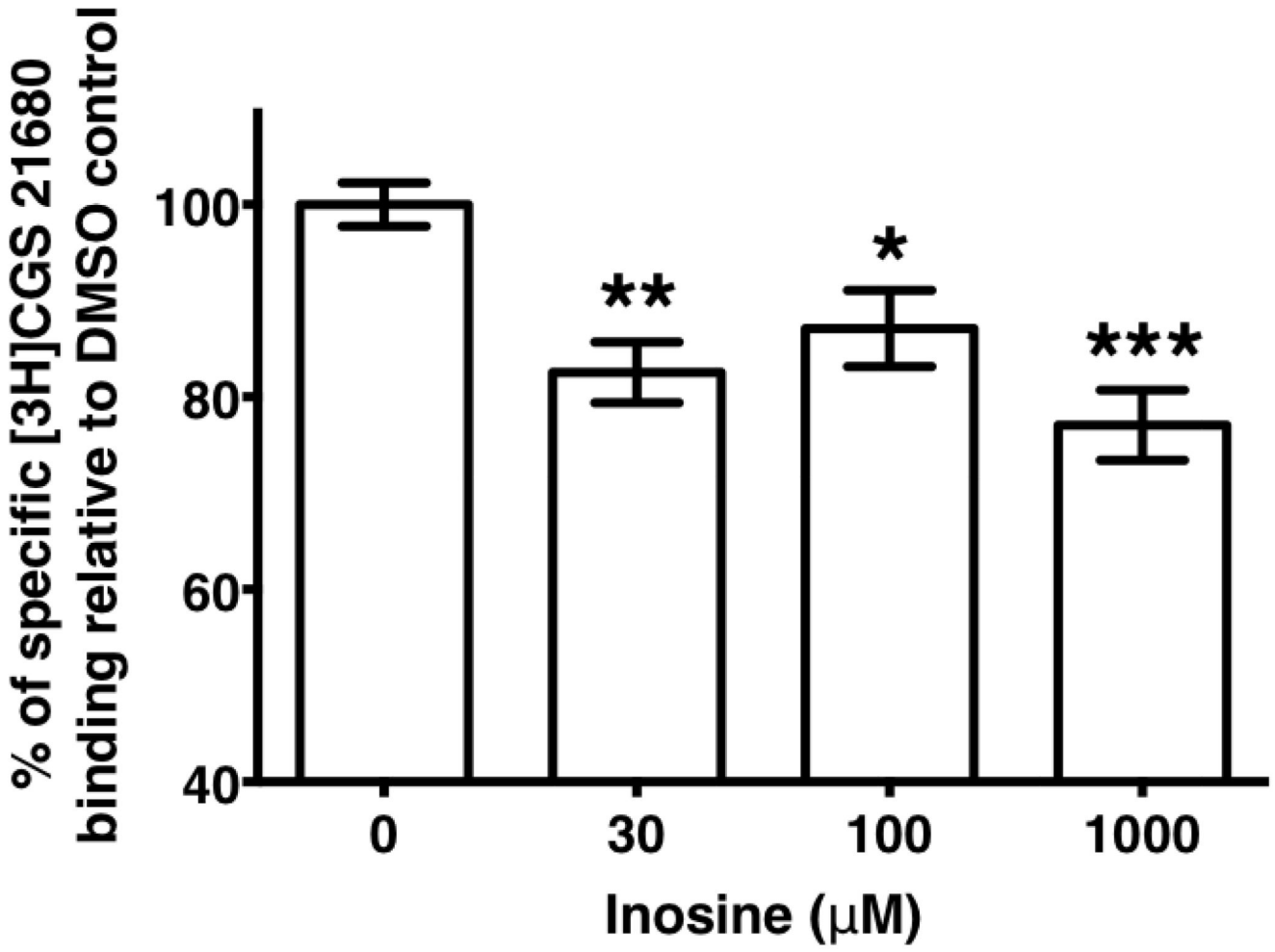


Fig. 8.

Displacement of $hA_{2A}R$-bound [^{3}H]CGS 21680 by inosine.

Equilibrium binding of [^{3}H]CGS 21680 to $hA_{2A}R$ in the presence of indicated concentrations of inosine and vehicle control. CHO-$hA_{2A}R$ membranes were incubated with ADA and [^{3}H]CGS 21680 in the presence and absence of inosine. Values are mean ± SEM of results of 3 independent experiments performed in triplicate; overall $p < 0.0001$; *, $p < 0.05$; ** < 0.01; *** < 0.001.

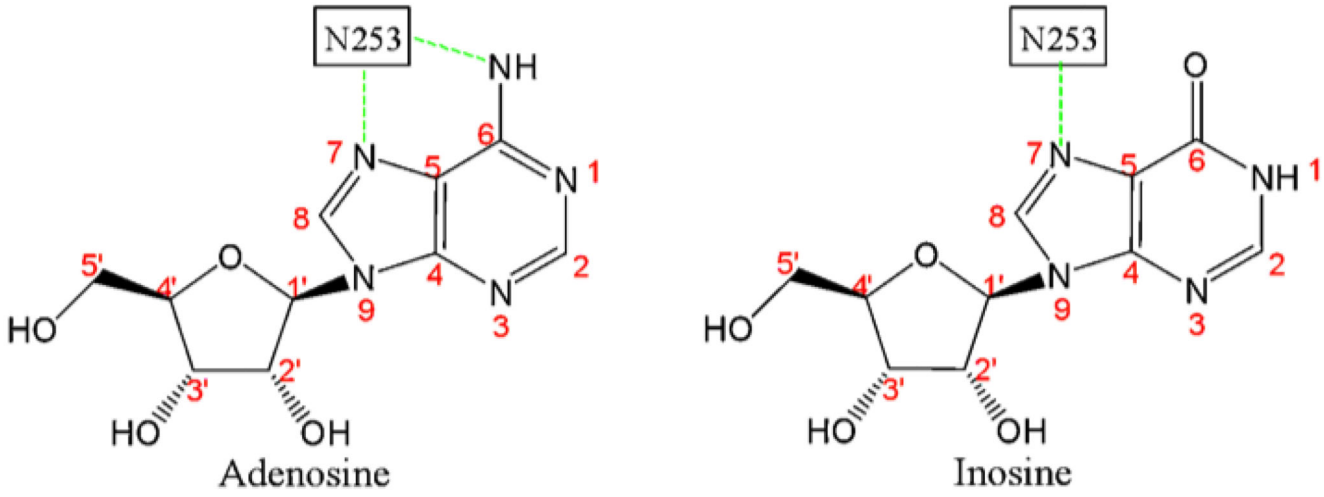


Fig. 9.

Schematic representation of hydrogen bonding interaction between adenosine/inosine and Asn^{253} in the sixth transmembrane domain of the $A_{2A}R$ agonist-binding pocket.

Table 1

Summary of EC_{50} ± SEM for adenosine- and inosine-stimulated cAMP production and ERK1/2 phosphorylation at the $hA_{2A}R$ and $mA_{2A}R$.

Agonist/ Inverse Agonist	EC_{50} (μM)		
	$hA_{2A}R$		$mA_{2A}R$
	cAMP Assay	ERK1/2 Phosphorylation Assay	cAMP Assay
Adenosine	0.006 ± 0.001	0.211 ± 0.036	0.059 ± 0.02
Inosine	300.7 ± 48.9	89.38 ± 15.12	709 ± 135.2
Adenosine + ZM 241385 (100 nM)	2.45 ± 1.29	1.17 ± 0.10	7.34 ± 4.56
Inosine + ZM 241385 (100 nM)	> 300	> 300	>1000